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Effect of Different Cultivation Models on Growth of *Haematococcus pluvialis* Flotow

Esra İMAMOĞLU* Meltem CONK DALAY Fazilet VARDAR SUKAN
Ege University, Faculty of Engineering, Department of Bioengineering, Izmir, 35100, Turkey

*Corresponding Author
e-mail: esraimamoglu@yahoo.com

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Abstract

The aim of this study was to investigate and compare the effects of different cultivation models in order to maximize the growth rate of different *Haematococcus pluvialis* Flotow strains for batch cultivations. After 12 days of growth period when compared to the effects of different cultivation models on the growth of different *H. pluvialis* strains in the Rudic's culture medium, the maximum cell concentrations of $1.1x10^6$ cells ml^{-1}, corresponding to the growth rate of 0.012 h^{-1}, was obtained in the autotrophic cultivation model under the light intensity of 75 µmol photons m^{-2} s^{-1} for *H. pluvialis* EGE MACC-35.

Key words: *Haematococcus pluvialis,* Heterotrophic cultivation, Autotrophic cultivation, Mixotrophic cultivation

Nomenclature

C_b	biomass concentration (g/l)
C_c	cell count (cells/ml)
DT	doubling time (h)
P_{bv}	volumetric productivity (g l^{-1} h^{-1})
µ	specific growth rate (h^{-1})

INTRODUCTION

The green unicellular fresh water microalga, *Haematococcus pluvialis* Flotow (Volvocales, Chlorophyceae) is green-colored, biflagellate, and motile in its vegetative stage [1]. This microalga shows low growth rates and low final cell densities under optimal growth conditions [2]. *Haematococcus* cells are sensitive to high hydrodynamic stress and changes in cell morphology under various environmental conditions [3]. No toxicity associated with *Haematococcus* has ever been reported in the literature. The general composition of *Haematococcus* algae consists of common carotenoids, fatty acids, proteins, carbohydrates, and minerals [4].

In recent years the green microalga *H. pluvialis* has been considered as a possible natural source for the production of astaxanthin and it has been widely studied. Astaxanthin is used as a pigment in aquaculture and food industries and has applications in pharmaceuticals and nutraceuticals due to its higher antioxidant activity than β-carotene and vitamin E. In addition, astaxanthin has important metabolic functions including enhancement of immune responses and protection against diseases such as cancer. Astaxanthin sells approximately \$2500 kg^{-1} in the worldwide aquaculture market [5]. *Haemetococcus pluvialis* is cultivated commercially by several companies. In Hawaii, outdoor closed photobioreactors as well as a combination of closed photobioreactors and open culture ponds are being successfully used to commercially produce *Haemetococcus* [6].

Algae are capable of many kinds of trophy (nourishment) centred on both major forms of nutrition, namely autotrophy (phototrophy) and heterotrophy, of which autotrophy is by far the most important. Autotrophic organisms obtain their energy through the absorption of light energy for the reduction of CO_2 by the oxidation of substrates, mainly water, with the release of O_2. Heterotrophic organisms obtain their material and energy needs from organic compounds produced by other organisms. Several algae species can be grown exclusively on organic substrates and this has become a viable obtain in conventional closed bioreactor production systems for biomass and biocompounds, produced by certain species under specific growth conditions. Mixotrophic organisms require light as energy source to use organic compounds as nutrients. The organic compounds may also satisfy the energy requirements of the algae. Mixotrophic growth is equivalent to autotrophy and heterotrophy, where both organic compounds and CO_2 are necessary for growth [7].

The aim of this study was to investigate and compare the effects of different cultivation models in order to maximize the growth rate of different *H. pluvialis* strains for batch cultivations.

MATERIALS AND METHODS

Algal strain and inoculum preparation

Haematococcus pluvialis Flotow EGE MACC-35 and *H. pluvialis* EGE MACC-36 were obtained from the Culture Collection of Microalgae at the University of Ege, Izmir, Turkey. Stock cultures of *H. pluvialis* strains were grown autotrophically in BG11 medium [8] at 25 °C under continuous illumination (100 µmol photons m^{-2} s^{-1}) in a 10-L flask. For the preparation of the inoculum, the cells from the stock culture were collected and concentrated by centrifugation (1,160 x g, 2 min) and the supernatant was

removed. The collected cells were transferred, incubated aseptically in a 1000 ml Erlenmeyer flask containing 800 ml of fresh BG11 medium under continuous illumination (75 µmol photons m^{-2} s^{-1}), at 25 °C for 4 days. Air was supplied to the culture at a flow rate of 1 L min^{-1} (1.25 vvm). 4-day old culture (at vegetative cell growth phase) was used as inoculum at 10% volume for all experiments.

Growth conditions

The temperature was measured in the centre of the flask with a thermocouple (Dixell-XT115), controlled by air conditioner. Illumination was provided by standard cool white fluorescent lamps (18 W) from one side of the flask. Irradiance was measured in the centre of the flask with a quantum meter (Lambda L1-185). Continuous aeration was provided by bubbling air, using a blower (Nitto Kohki Co, LTD). Rotameters (Özgül-air) were used to provide the desired air flow rate. Pure CO_2 was added intermittently, using timer (Akboru BND-50/G1), for 10 s every 10 min to the air stream (1.5% v/v) in order to provide inorganic carbon to the culture and keep the pH value below 8. Styrofoam was used to avoid the light penetration between the experiments when studies dealing with the different light intensities.

The experiments were performed in 1000 ml Erlenmeyer flasks. 4-day old culture (approximately 2 x 10^6 cells ml^{-1}) was inoculated into 800 ml Rudic's medium [9] in 1000 ml Erlenmeyer flasks. Air was supplied to the culture at a flow rate of 1 L min^{-1}(1.25 vvm). The flasks were incubated for 12 days at 25 °C. The compositions of BG11 and RM culture media were shown in Table 1. If necessary, the vitamins were added aseptically to the final medium after autoclaving. All components (Merck Co.) were used analytical grade.

Table 1 Composition of BG11 and RM culture media

Constituents	BG11 (mg/L)	RM (mg/L)
$NaNO_3$	1500	300
K_2HPO_4	40	80
KH_2PO_4	-	20
$MgSO_4.7H_2O$	75	10
$CaCl_2.2H_2O$	36	58.5
Citric Acid	6	-
Ammonium Ferric Citrate	6	-
EDTA-Na_2	1	-
EDTA	-	7.5
Na_2CO_3	20	-
NaCl	-	20
H_3BO_3	2.86	0.3
$MnCl_2.4H_2O$	1.81	-
$MnSO_4H_2O$	-	1.5
$ZnSO_4.7H_2O$	0.22	0.1
$Na_2MoO_4.2H_2O$	0.39	-
$(NH_4)6Mo7O_{24}.4H_2O$	-	0.3
$CuSO_4.5H_2O$	0.08	0.08
$Co(NO_3)_2.6H_2O$	0.05	0.26
$FeCl_3.6H_2O$	-	17

Cultivation Models

Different cultivation models are formed using the metabolic properties of *Haematococcus pluvialis.* These are autotrophic**,** mixotrophic and heterotrophic cultivations. Autotrophic cultivation was achieved in the presence of light and supplied with CO_2. Mixotrophic cultivation was applied in the presence of light and supplied with Na-acetate. Heterotrophic cultivation is achieved under the dark conditions and supplied with Na-acetate [10]. In this context, 1 g/L Na-acetate and the light intensity of 75 µmol photons m^{-2} s^{-1} were used in this study.

Analytical procedure

Samples were taken at indicated times, and following growth parameters were measured immediately; the cell concentration was determined by counting triplicate samples in a Neubauer haemocytometer. Dry weight was determined in duplicate by filtering a 5-mL culture sample through preweighed Whatman GF/C filters and drying the cell mass at 105 °C for 2 h. The correlation between the cell count (C_c) and the biomass concentration (C_b) was: C_b (g l^{-1}) = 0.0668 C_c (10^5cell/ml) + 0.1304, where C_b is the dry weight biomass concentration.

The specific growth rate (µ) of the cells was calculated from the initial logarithmic phase of growth for at least 48 h, as $\mu = (\ln C_{c2} - \ln C_{c1})/\text{delta t}$, where C_{c2} is the final cell concentration, C_{c1} is the initial cell concentration and delta t is the time required for the increase in concentration from C_{c1} to C_{c2}. Doubling time (DT) was also calculated as $DT = \ln 2/\mu$. The volumetric productivity of the biomass is calculated [11]: $P_{bv} = \mu C_b$, where P_{bv} is the volumetric productivity and C_b is the concentration of the biomass.

RESULTS

As shown in Figure 1, after 12 days of growth period when compared to the effects of different cultivation models on the growth of *H. pluvialis* EGE MACC-35 in the Rudic's culture medium, the maximum cell concentrations of 1.1x10^6 cells ml^{-1}, corresponding to the growth rate of 0.012 h^{-1}, was obtained in the autotrophic cultivation model under the light intensity of 75 µmol photons m^{-2} s^{-1}. On the other hand, there was a lower increase in mixotrophic model with the value of 4.7x10^5 cells ml^{-1}, which corresponds to the growth rate of 0.009 h^{-1}, compared to autotrophic cultivation at the light intensity of 75 µmol photons m^{-2} s^{-1}. For the heterotrophic cultivation model, the cell concentration of 1.7x10^5 cells ml^{-1} (the growth rate of 0.006 h^{-1}) was obtained on the 3rd day of cultivation period, then occurred 2.5 times of cell death until the day of 7, after 8 days of cultivation period, the curve nearly remained at constant levels. When taken into consideration the growth rates of the cultivation models, the growth increased 2.20 times in autotrophic cultivation model and 1.50 times in mixotrophic cultivation model with respect to the heterotrophic cultivation model for the strain of *Haematococcus pluvialis* EGE MACC-35.

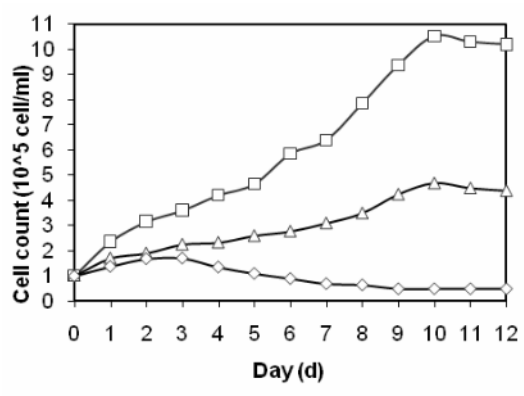

Figure 1. Effect of different cultivation models on growth of *H. pluvialis* EGE MACC-35. (□) Autotrophic, (Δ) Mixotrophic, (◊) Heterotrophic.

The maximum cell count, 5.1x 10^5 cells ml^{-1}, was obtained in the autotrophic cultivation model under the light intensity of 75 µmol photons m^{-2} s^{-1} and the lowest in the heterotrophic cultivation model (1.6 x 10^5 cells ml^{-1}) for the strain of *H. pluvialis* EGE MACC-36 (Figure 2). The cell concentration of 1.9x10^5 cells ml^{-1}, which corresponds to the volumetric productivity of 0.002 g l^{-1} h^{-1}, was reached in the mixotrophic cultivation model after 12 days of growth period (Table 2). It should be noted here that when the volumetric productivity decreased due to the dilute culture broth, this could have caused an increase in the cost of recovering the biomass.

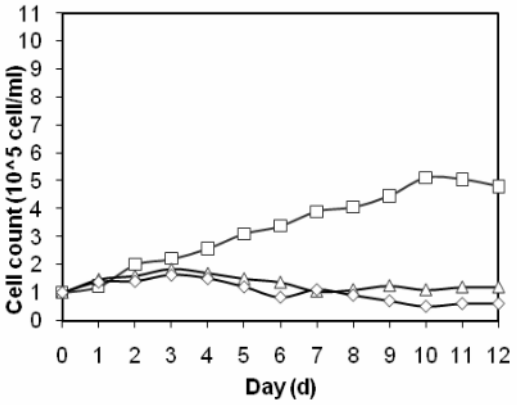

Figure 2. Effect of different cultivation models on growth of *H. pluvialis* EGE MACC-36. (□) Autotrophic, (Δ) Mixotrophic, (◊) Heterotrophic.

Table 2. Results of obtaining kinetic parameters of different *H. pluvialis* strains produced in different cultivation models

H. pluvialis **Ege-MACC-35**	C_b (g l^{-1})	µ (h^{-1})	DT (h)	P_{bv} (g l^{-1} h^{-1})
Autotrophic	0.833	0.012	57	0.010
Mixotrophic	0.444	0.009	82	0.004
Heterotrophic	0.244	0.006	124	0.002
H .pluvialis **Ege-MACC-36**				
Autotrophic	0.471	0.009	74	0.004
Mixotrophic	0.254	0.007	94	0.002
Heterotrophic	0.239	0.005	151	0.001

When the strains of *H. pluvialis* were considered, the growth increased by 90% for the strain of *H. pluvialis* EGE MACC-35 with respect to the strain of *H. pluvialis* EGE MACC-36 in the autotrophic cultivation model under the same light intensity. When taken into consideration the growth rates of the cultivation models, the growth was the same in mixotrophic cultivation model of *H. pluvialis* EGE MACC-35 and in autotrophic cultivation model of *H. pluvialis* EGE MACC-36 (Table 2).

DISCUSSION

Several experiments have been carried out by different authors on the growth of *Haematococcus* sp. in order to obtain maximum growth rate. It is rather difficult to compare the results due to differences in growth conditions and strains for microalgal biotechnological applications. As reported by Margalith P. (1999), the specific growth rate was 0.013 h^{-1} in autotrophic cultivation model, 0.024 h^{-1} in mixotrophic cultivation model and 0.009 h^{-1} in heterotrophic cultivation model [10].

The very cheap method of growing *H. pluvialis* in open ponds will not materialize because of the contamination problems. The mixotrophic mode of growth in closed systems should be the basis for further developments and advanced fermentation technology, possibly by well-regulated alterations between light-independent mass cultivation and light-dependent carotenogenesis in the algal life cycle [10].

Light is essential for the life cycle of *H. pluvialis*. Higher light intensities can lead to photoinhibition. Light penetration (which is inversely proportional to cell concentration) is another problem in the phototrophic cultivation of microalgae [10]. Under optimal growth conditions, light absorbed by antenna pigments is converted to chemical energy forming ATP and NADPH through a photosynthetic electron transport chain. This chemical energy is finally stored in starch by fixing CO_2 through the Calvin cycle [12].

In mixotrophic cultures of *Haematococcus lacustris*, photosynthesis and oxidative metabolism of acetate functioned simultaneously [13]. Despite only meager cell densities obtained in the heterotrophic batch culture, this mode of growth is considered to be important since large scale high cell density cultivation is possible in theory as light is not limiting factor under heterotrophic conditions [14]. The major problem with the heterotrophic cultivation of *Haematococcus* is contamination by bacteria and other heterotrops. Since the growth rate of *Haematococcus* is rather very low, adequate measures should be taken to prevent contamination. Even in a sterilized bioreactor, it was very difficult to maintain a pure culture for a long time [15].

For some kinds of algal strains, photosynthesis and oxidation of organic substrates proceed independently in an additive manner under mixotrophic condition so that the growth rate of cells in mixotrophic condition is equal to the sum of those in photoautotrophic and heterotrophic cultures [16]. This has been reported for several strains, such as *Chlorella vulgaris* [17], *Spirulina platensis* [18] and *Haematococcus pluvialis* [13].

Previous studies on the nutrition of *H. pluvialis* have shown that acetate is an important carbon source, enhancing both growth and carotenogenesis [19]. However the effect of acetate depends on its concentration, the growth inhibition is achieved at higher concentrations. It could be possible that the growth rate is increased by arranging the optimal concentration of organic carbon and the intensity of light under axenic conditions.

CONCLUSION

This study has demonstrated that the maximum cell concentrations of 1.1x10^6 cells ml^{-1}, corresponding to the growth rate of 0.012 h^{-1}, was obtained in the autotrophic cultivation model under the light intensity of 75 µmol photons m^{-2} s^{-1} for the strain of *H. pluvialis* EGE MACC-35.

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